# Molecular identification of babesiosis in stray dog at Chittagong Metropolitan Area

**Md. Nur-E-Azam**

Roll No. 0213/05

Registration No. 0153

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**Department of Pathology and Parasitological**

**Faculty of Veterinary Medicine**

**Chittagong Veterinary and Animal Sciences University**

**Chittagong-4225, Bangladesh**

**DECEMBER, 2014**

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**December, 2014**

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**This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made**

**(Dr. Mohammad Alamgir Hossain )**
Professor
Department of Pathology and Parasitology
**Supervisor**

**(Dr. Mohammad Mahbubur Rahman)**
Associate Professor
Department of Pathology and Parasitology
**Co- Supervisor**

**(Dr. Md. Masuduzzaman)**
Professor and Head
Department of Pathology and Parasitology
**Chairman of the Examination Committee**

**Department of Pathology and Parasitology**
**Faculty of Veterinary Medicine**
**Chittagong Veterinary and Animal Sciences University**
**Khulshi, Chittagong -4225, Bangladesh**

**DECEMBER, 2014**

DEDICATED TO MY BELOVED PARENTS AND YOUNGER BROTHER

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*The author*

# List of Abbreviations and Symbols

| | |
|---|---|
| < | = Less than |
| × | = Multiply |
| % | = Percentage |
| DNA | = Deoxyribonucleic Acid |
| RNA | = Ribonucleic Acid |
| CVASU | = Chittagong Veterinary and Animal Sciences University |
| ELISA | = Enzyme Linked Immuno-Sorbent Assay |
| PRTC | = Poultry Research and Training Centre |
| EDTA | = Ethylene diamine tetra acetic acid |
| PCI | = Phenol chloroform Isoamylalcohol |
| ITS | = Internal Transcribed Spacers |
| USA | = United States of America |
| CVH | = Central Veterinary Hospital |
| ID | = Identification |
| IFAT | = Indirect Immunofluorescence Antibody Test |
| IgG | = Immunoglobulin G |
| LAMP | = Loop mediated Host Isothermal Amplification method |
| PCR | = Polymerase Chain Reaction |
| RFLP | = Restriction Fragment Length Polymorphism |
| FAO | = Food and Agricultural Organization |
| N | = Sample size |

## Summary

An epidemiological study of babesiosis in stray dogs was conducted at Chittagong Metropolitan area, Bangladesh, for six months. Blood samples of 130 stray dogs were collected from nine randomly selected locations along with the information of age and sex. Blood samples were initially examined by Giemsa's stained blood smear method. DNA was extracted from microscopy positive blood samples and *Babesia* spp were confirmed by amplifying 18S rRNA gene. The prevalence was determined 6.92% in microscopic technique and the percentages of PCR positive samples from the microscopy positive samples were 66.67% (6 out of 9). The highest prevalence of babesiosis was found in Alankar (16.67%) compared to other areas. Significantly higher frequency of babesiosis was found in adult dog than younger ($p<0.05$). Again, *Babesia* infection was common in male (11.94%) than female (1.59%) and were statistically significant ($p<0.05$). Furthermore, we sought to develop a seminested PCR to detect and differentiate *Babesia gibsoni* (Asia genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis* and *B. canis* subsp. *rossi* DNA in canine blood samples. An outer primer pair was designed to amplify a ~340 bp fragment of the 18S rRNA genes in the seminested primary reaction. Then specific internal primers were designed for *B. gibsoni* (Asian type) (BgibAsia-F) and *B. canis* subsp. *canis* (BCC-F) that were paired with the outer reverse primer in the seminested secondary reaction to amplify 185 bp and 198 bp respectively. Based on seminested PCR and sequencing of the PCR products, all parasitic isolates were identified as *Babesia gibsoni* (Asian type) and *B. canis* subsp. *canis*. These results recommended that *Babesia gibsoni* (Asian type) and *B. canis* subsp. *canis* parasites are widespread in stray dog in Chittagong Metropolitan area, Bangladesh. Further investigations are required to clarify the origin, distribution, vector and pathogenesis of these parasites tracking in dogs in Bangladesh.



**Key words:** Stray dog, 18S rRNA gene, *Babesia gibsoni* (Asia genotype), *B. canis* subsp. *canis*, Seminested PCR, Sequencing

# Chapter I: Introduction

Bangladesh has a wide range of climatic region, from plane and costal to the hilly area, which make it suitable for a diverse range of vectors and pathogens of medical and veterinary importance, whose transmission and geographical distribution are closely linked to regional temperature, rainfall and humidity (Rahman, 2014).

The vector borne parasitic diseases affect not only human but also the domestic, wild and companion animals. Knowledge of parasitic diseases of companion animals in Indian subcontinent remains incomplete, despite climatic conditions that are often conducive for the transmission of enteric and vector-borne parasitic infections (Rani et al., 2010). Dog population can be divided into four categories which can be defined as follows: pets (restricted and supervised); family dogs (partially restricted, wholly dependent); community dogs (unrestricted, partially dependent); and feral or stray dogs (unrestricted, independent) (Menezes, 2008).

Stray dogs are unconfined dogs that live virtually wherever in the cities and the local human population exist (Daniels, 1983; Das et al., 2011). They act as the usual connectors between people and nature (Beck, 1973) and contributing high risk of parasitic zoonoses (Khante et al., 2009). In fact dogs are associated with >60 zoonotic diseases among which can pose serious health concern, as well as significant economic impact from veterinary standpoint (Ranjbar-Bahadori et al., 2008; Das et al., 2012).

Parasitism is the most commonly encountered disease in dogs all over the world (Traub, 2003). Again Dogs are competent reservoir hosts of several hemopathogens including zoonotic agents and can serve as readily available source of nutrition for many blood-feeding arthropods (Qablan et al., 2012). Developing countries like Bangladesh, the number of stray dogs that coexist with human being is high in most cities and villages which constitute a potential risk of infections for human beings. The distribution and intensity of parasitism in dogs are influenced by geographical, climatic, cultural and economic factors (Robertson et al., 2000; Das et al., 2012).

Amongst the various prevalent canine vector-borne diseases, canine babesiosis is very common and clinically significant disease caused by intraerythrocytic apicomplexan protozoa belonging to genus *Babesia* (Singh et al., 2014). Canine babesiosis, an important tick-borne infectious disease of dogs, has been described as an emerging veterinary problem worldwide (Duh et al., 2004; Irwin, 2009). The increasing number of canine Babesia species, geographical distribution, varying tick vectors and modes of transmission often result in diverse of pathogenic and clinical presentations (Ayoob et al., 2010).

*Babesia* species often referred to as piroplasms comprise two main species, *B. canis and B. gibsoni*, based on their size. B. canis is a large piroplasm (4-5 $\mu$m), which usually occurs as a single pear-shaped piroplasm or in pairs of merozoites divided by binary fission within the erythrocyte (Birkenheuer, 2004; Ahmad et al., 2007; Bashir, 2008; Irwin, 2010).

Previous studies, on the basis of differences in the geographical distribution, vector specificity, and antigenic properties (Uilenberg et al., 1989; Hauschild et al., 1995), recognized that large canine piroplasms are subdivided into three species, namely, *B. canis* transmitted by *Dermacentor reticulatus* (in Europe), *B. vogeli* transmitted by *Rhipicephalus sanguineus* (in tropical and subtropical regions), and *B. rossi* transmitted by *Haemaphysalis elliptica* (in South Africa). *B. gibsoni* has been found to be associated with infection of dogs in Asia, North America, northern and eastern Africa, and Europe (Conrad et al., 1991; Casapulla et al., 1998; Birkenheuer et al., 1999). It is a small parasite that commonly appears as individual ring forms or pyriform bodies ranging between 1.0 and 2.5 $\mu$m in size (Conrad et al., 1991).

As far as the diagnosis of canine babesiosis is concerned, direct microscopic examination of the stained blood smear is the most commonly used method as it is conclusive, feasible, and cost effective diagnostic method but not necessarily detects parasites in dogs with unapparent or chronic infections since the level of parasitemia is very low (Cacciò et al., 2002). As regards, the serological methods, indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) for *B. gibsoni* parasites, are considered to be highly sensitive, but only moderately specific because of antigenic cross-reactions to *B. canis* (Yamane et al., 1993a) and normal dog erythrocytes (Yamane et al., 1993a; Adachi et al., 1994). Therefore, the

development of highly specific and sensitive system for the diagnosis of canine babesiosis is still awaited. In this regard, recent advances in molecular biology techniques like polymerase chain reaction (PCR) have made it possible to detect and identify piroplasms with greater sensitivity and specificity than traditional methods (Birkenheuer et al., 2003b; Jefferies et al., 2009; Salem and Farag, 2014; Singh et al., 2014).

Regarding Bangladesh scenario, though there are sporadic reports of canine babesiosis based on conventional diagnostic methods (Tarafder and Samad, 2012; Mahmud et al., 2014). Again the true status of canine babesiosis is still not clear barring only two reports (Talukder et al., 2013; Terao et al., 2015) employing the PCR based assays. Currently, there are no data available on the distribution, prevalence, parasitic burden and risk factors associated with babesiosis of stray dogs in Chittagong. Furthermore, molecular detection of canine babesiosis has not yet been explored from Chittagong of Bangladesh, so the present work was carried out to know the status of canine babesiosis in this part of the country through PCR based assays. This study will assist the policy maker to take effective preventive and control measures against this disease.

### 1.1 Objectives of the study

- To know the prevalence of babesiosis in stray dog at Chittagong Metropolitan area of Bangladesh.
- Standardization of PCR-based molecular diagnosis and identification of babesiosis.
- Capacity building for genotyping/subgenotyping of *Babesia* spp at Chittagong Veterinary and Animal Sciences University (CVASU).
- To determine the effect of different risk factors such as age, sex, location etc. in the occurrence of such disease.

# Chapter II: Review of literature

Babesiosis is a dangerous, invasive disease of humans and animals. Victor Babes in 1888 described the first intra-erythrocytic piroplasms in cattle. Subsequently in 1893 Smith and Kilbourne, (cited by Birkenheuer, 2004) reported that the causative agent of Texas fever in cattle was transmitted by ticks and named the causal agent as *Pyrosum bigemina* (believed to be either *Babesia bigemina* or *Babesia bovis*). They acknowledged the babesiosis as the first tick-transmitted disease. Hutcheon, (cited by Birkenheuer, 2004) was the one who reported the presence of intracellular parasities in cases of canine babesiosis in 1896 in South Africa. *Babesia canis* was first described by Piana and Galli-Valerio in 1895 and was historically recognized as the only species of large piroplasm known to infect dogs. In 1901 Lousby (cited by Birkenheuer, 2004) documented *Haemaphysalis leachi* to be the vector for *B.canis rossi*. Patton in 1910 was first researcher to explain a small piroplasm infecting canids and named the organism as *Piroplasma gibsoni* in honour of Dr. F. Maitland Gibson who first saw the parasite. Ristic and Kreier (1984); Levine (1988) and Ristic (1988) described that the *Babesia* spp. are the intraerythrocytic protozoan parasite belonging to the phylum Apicomplexa and are transmitted by ticks. Members of the order Piroplasmidia are apicomplexan protozoa categorized into four main families; Anthemosomatidae, Babesiidae, Theileridae and Haemohormidiidae (Levine, 1988). By now more than 100 species have been phenotypically documented (Ristic and Kreier, 1984; Levine, 1988; Ristic, 1988). Historically the families Babesiidae and Theileriidae were documented to include genra, *Babesia, Cytauxzoon* and *Theileria.* Historically, multiple genus names have described the Piroplasmidia including *Piroplasma, Pyrosoma, Apisoma, Nuttallia, Nicollia, Babesiosoma, Smithia* and *Rossiella* (Levine, 1988).

## 2.1 History of babesiosis in Bangladesh

In 1959, Abdussalam reported that the Indo-Pakistan Sub-continent, with its varied topography and climatic conditions, is rich in tick fauna which are widely distributed in this region (cited by Bashir, 2008). In buffaloes, cattle, sheep, goats, equines and dogs babesiosis results in loss of life and serious ill health with consequent loss of

working and productive capacity. He further documented the occurrence of *Babesia gibsoni* in dogs and jackals throughout Indo-Pakistan. Samad et al. (1989); Chowdhury et al. (2006); Siddiki et al. (2010) and Alim et al. (2012), all of them conducted research on babesiosis in cattle in different parts of Bangladesh. Talukder et al. (2013) conducted a PCR-based survey of vector borne pathogens in dog in Dhaka and examined 68 suspected blood samples, from which they found 26 positive for *Babesia gibsoni.* Tarafder and Samad (2012*)* reported the prevalence of canine babesiosis in Bangladesh as 0.08%. A case control study was conducted to ascertain the prevalence of clinical diseases and/or clinical conditions of 3670 sick pet dogs presented to the Central Veterinary Hospital (CVH), Dhaka during the one year period from January to December 2009. Terao et al. (2015) found *Babesia gibsoni* in dog of Dhaka city.

## 2.2 Classification

Since the earliest research started, *Babesia* spp. have been classified on the basis of their size and vertebrate host (Ristic et al., 1982; Euzeby, 1987; Levine, 1988; Ristic, 1988). Levine (1988) described that *Babesia* are classified in the Phylum Apicomplexa, Class Sporozoae, Order Piroplasmida, and family Babesiidae. The family Babesiidae contains three genera, *Babesia, Theileria* and *Cytauxzoon*. The absence of a preerythrocytic stage in the life cycle within the animal host is considered an important phenotypic feature of the genus *Babesia*. *Babesia* greater than 3 microns in length and between 1-3 microns in length are considered to be "large" and "small" *Babesia,* respectively (Ristic, 1988). The knowledge of phylogenetic relationships between the piroplasms became clear by the use of genetic-based analysis. Escalante and Ayala (1995) stated that a few studies have proposed the overall phylogenetic relationships between members of the phylum Apicomplexa and is likely to be the reflection of the many thousands of species described.

Early classification was mainly based on the morphology and lifecycle of the organism. Mehlhorn and Schein (1985) reported that the members of the genus *Theileria* were differentiated from other species of piroplasm by the presence of a tetrad or 'maltese cross' formation of the intraerythrocytic merozoites and the existence of an exoerthrocytic lifecycle stage and also differentiated by transatadial transmission in the tick.

### 2.2.1 Large canine piroplasm species

Piana and Galli-Valerio (1895) described *Babesia canis* for the first time and was historically recognized as the only species of large piroplasm known to infect dogs cited by (Jefferies et al., 2009). Since then at least five different species or sub-species of large *Babesia* have been described in dogs. The differences in the pathology and vector specificity among the isolates categorized them into three different sub-species of *Babesia canis* (Uilenberg et al., 1989). The transmission of *Babesia canis canis, Babesia canis vogeli* and *Babesia canis rossi* are by *Dermacentor* spp., *Rhipicephalus sanguineus* and *Haemophysalis leachi,* respectively. *Babesia canis rossi* is responsible for producing the major severe disease and *Babesia vogeli* the least pathogenic. Carret et al., (1999) confirmed the three subspecies, by DNA sequencing of 18S rRNA, whereas (Zahler et al., 1998) used a different gene locus of the Internal Transcribed Spacers (ITS) 1 and 2. These authors further stated that the three subspecies are genetically distinct enough to obtain species status in their own right (Zahler et al., 1998; Carret et al., 1999). *Babesia canis presentii* was reported in domestic cats from Israel (Baneth et al., 2004) and a fifth large canine *Babesia* has been reported in a Labrador in North Carolina (Birkenheuer et al., 2004).

### 2.2.2 Small canine piroplasm species

Patton was the first to describe the *Piroplasma gibsoni* as a species when this small piroplasm was identified within the blood of dogs and jackals in India. It was renamed as *Babesia gibsoni* on the basis of shape, size and host specificity (Botros et al., 1975). Another small piroplasm was reported by Zahler et al. (2000b) that was genetically similar to rodent/human species, *B.microt*i, and distinct from *B. gibsoni* on the basis of the 18S rRNA gene. On the basis of the sequence analysis of the 18S rRNA gene, isolates of small canine *Babesia* from Asia and North America were also compared (Zahler et al., 2000c). From the over 100 listed *Babesia* species, genetic data for only 15 species is listed in GENBANK. Benson et al. (2003) documented that there was a distinct genetic separation between the large and small *Babesia* species (Homer et al., 2000).

**Table 1:** List of Established and Molecular Characterized Canine Species

| Piroplasm size | Established canine species | Molecular characterized canine species |
|---|---|---|
| Large (3 – 5 mm) | *Babesia canis* | *Babesia canis canis* (Carret et al., 1999)<br>*Babesia canis vogeli* (Carret et al., 1999)<br>*Babesia canis rossi* (Carret et al., 1999)<br>*Babesia* sp. (Birkenheuer et al., 2004) |
| Small (1 – 2 mm) | *Babesia gibsoni* | *Babesia gibsoni* (Zahler et al., 2000c)<br>*Babesia conradae* (Kjemtrup et al., 2000a) |

### 2.3 General phylogenetic classification

Phylogenetic and evolutionary relationships among few species of *Babesia, Theileria* and *Cytauxzoon* were first assessed by Allsopp et al. (1994) on the basis of the 18S rRNA gene and reported *Babesia* and *Theileria* species separately into distinct monophylectic clades and also inferred a third group comprising *Babesia rodhaini*, *Babesia equi* and *Cytauxzoon felis*, and proposed a new family *Nicollidae*. Kjemtrup et al. (2000b) inferred four groups from phylogenetic trees. These groups were *Babesia sensu stricto*, *Theileria*, Western *Babesia* species (contained wildlife and human piroplasm spp.) and *Babesia microti* (ancestral to all three groups of piroplasms).

In early analysis the phylogenetic position of canine piroplasms related to *B.canis*. Allsopp et al. (1994) documented that *B.canis* belonged to the *B. sensu stricto* group, and it became more evident when Carret et al. (1999) documented that *B.canis canis* and *B.canis rossi* cluster together and *B.canis vogeli* separated into a monophyletic group with *B.odocoilei* and *B. divergens* (Criado-Fornelio et al., 2003a).


**Figure 1:** Phylogenetic tree of the Piroplasmida on distance basis

**Adapted from** (Criado-Fornelio et al., 2003a), Arrows indicate piroplasm species found in dogs.

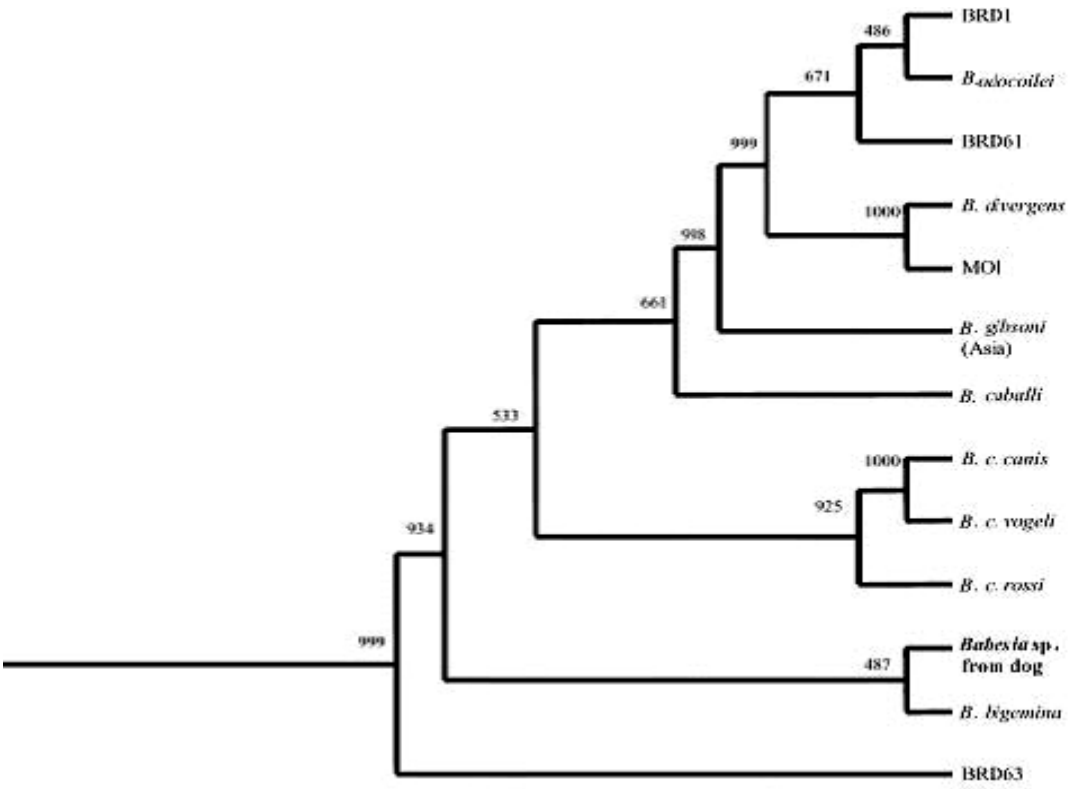


**Figure 2:** Partial phylogenetic tree identifying the evolutionary relationships between the large *Babesia* sp. from a dog (North Carolina) and other *Babesia* species

**Adapted from** (Birkenheuer et al., 2004)

## 2.4 Biology

The *Babesia* species are one of the most ubiquitous and widespread blood parasites in the world based on numbers and distribution of species in animals, second to the trypanosomes (Levine, 1988; Quick et al., 1993). They generally have two classes of hosts, an invertebrate and a vertebrate. The maintenance of *Babesia* spp. is dependent on both hosts; the specific tick vector must feed on a vertebrate reservoir that is competent in maintaining the *Babesia* organisms in an infectious state. Therefore, *B. microti* presents itself as an emerging zoonosis only in areas where there is a primary competent reservoir (Homer et al., 2000). *Babesia* species can be found wherever certain species of ticks flourish. To date, only Ixodid ticks have been identified as vectors for *Babesia* spp. except for one report that identified a non- Ixodid tick, *Ornithodoros erraticus*, as a reservoir for *Babesia meri*. Six of the seven main genera of Ixodid ticks have been demonstrated as experimental or natural vectors of diverse *Babesia* species.

## 2.5 Morphology

### 2.5.1 Large canine piroplasm species

Almost all large piroplasms of dogs are 2-5µm in diameter. The length and width of trophozoite of *B.canis* is 5.0µm and 2.5-3.0µm, respectively and as previously noted are commonly known as large canine *Babesia* (Kuttler and Aliu, 1984; Conrad et al., 1992). *Babesia canis* are generally pear-shaped, occurring singularly or as pairs of dividing trophozoites inside the erythrocyte (Kjemtrup et al., 2000a). In contrast, Birkenheuer et al. (2004) documented a new *Babesia* spp. (North Carolina) as polymorphic, typically singular, with occasional two pyriform shaped organisms joined at a 90° angle. The size ranges from 2µm x 3.5µm to 5µm x 6µm.


**Figure 3:** Typical morphology of 'large' canine piroplasms (*Babesia canis vogeli*).

**Adapted from** (Jefferies, 2001)

### 2.5.2 Small canine piroplasm species

The small piroplasms are typically 0.5-3µm in diameter. As the trophozoite of *Babesia gibsoni* are small 1.2 to 2.2µm in size and are referred as small canine *Babesia* (Kuttler and Aliu, 1984; Casapulla et al., 1998; Fukumoto et al., 2001). *Babesia gibsoni* also appear as pleomorphic protozoa, usually round, oval or pear shaped (Conrad et al., 1992; Casapulla et al., 1998). *Babesia gibsoni* mostly occurs in singles but may occur in paired forms occasionally in red blood cells (Fukumoto et al., 2001). Kocan et al. (2001) reported that *B.conradae* and *T.annae* are capable of forming intraerythrocytic tetrads.

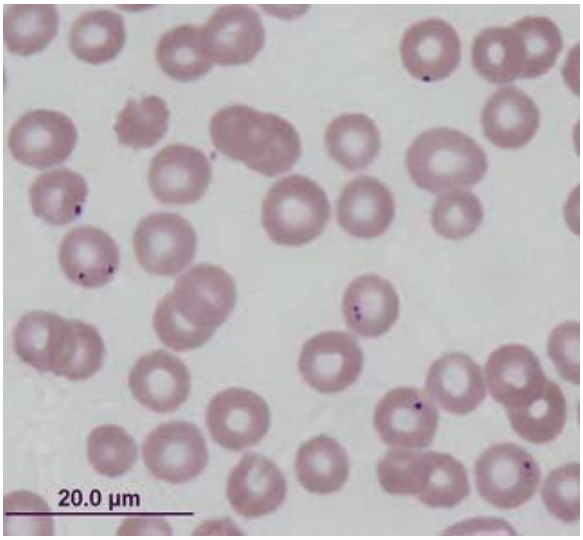


**Figure 4:** Morphology of small canine piroplasm *Babesia gibsoni*

**Adapted from** (Bashir, 2008)

## 2.6 Transmission

### 2.6.1 Tick/Vector Transmission

Within the invertebrate host, both transovarial and transstadial transmission of *Babesia* can occur (Ristic et al., 1982). Transovarial transmission is characterized by the ability of an infected tick to produce progeny (larvae and nymph) that are infectious. Transstadial transmission is characterized by the ability of an individual tick to retain infectivity throughout multiple stages of its lifecycle.

Canine *Babesia* is transmitted by at least three different genera of Ixodid ticks. *Babesia canis canis* is transmitted by *Dermacentor reticulatis*. *Babesia c. vogeli* is transmitted by *Rhipicephalus sanguineus*. *Babesia c. rossi* is transmitted by *Haemaphysalis leachi* (Uilenberg et al., 1989). *Babesia gibsoni* (Asian genotype) is transmitted by *Haemaphysalis bispinosa* and *Haemaphysalis longicornis* (Swaminath and Shortt, 1937; Otsuka, 1974).

*Babesia gibsoni* has been found in the blood of young puppies and their dams, indicating that transplacental transmission may occur (Harvey et al., 1988) and Fukumoto et al. (2005) suggested that transplacental transmission is now proved experimentally.

### 2.6.2 Direct transmission (Non-vector)

Blood–borne pathogens can be transmitted by blood transfusion both in human and veterinary medicine (Herwaldt et al., 2002; Kjemtrup et al., 2002; Powell and Grima, 2002; Cable and Leiby, 2003; Leiby and Gill, 2004). Wittner et al. (1982) documented first transmission of babesiosis in a patient when he received blood infected with *B.microti.*

Wardrop et al. (2005) emphasized on the need to screen potential blood donor dogs, as two species of canine *Babesia* are reported to be transmitted through blood transfusion. *Babesia gibsoni* has been reported to be transmitted during a whole blood transfusion, with the donor blood originating from an American Pit Bull Terrier (Stegeman et al., 2003). Jacobson and Clark (1994) also noted transfusion associated transmission in *B.canis rossi* infections.

The direct blood to blood transmission has been reported in breeds used in dog fighting. A high prevalence of *B.gibsoni* has been described in American Pit Bull Terriers in the USA (Birkenheuer et al., 1999; Macintire et al., 2002; Birkenheuer et al., 2003b). Matsuu et al. (2004) also reported the high prevalence of *B.gibsoni* in Tosa dogs in Japan. It has been postulated in both countries that direct blood-to-blood transmission of *B.gibsoni* may occur during biting or fighting between dogs. Matsuu et al. (2004) also speculated that transmission of the parasite may occur during mating.

The movement of family-owned and military working dogs between countries is also a major contributing factor in increased distribution of canine piroplasmosis (Anderson et al., 1980; Shaw et al., 2001). The movement of chronically ill animals into disease free areas is a big source of spread of *B.gibsoni* in USA and it is also hypothised that military dogs returning from Japan were responsible for the original introduction of this parasite into the USA (Anderson et al., 1980).

An important feature of piroplasm infection is the facilitation of wild canids as reservoirs of these parasites (Jefferies, 2006). Many canine species have been described as potential hosts for canine *Babesia* throughout many regions of the World. Jackals in India, foxes, jackals and fenece in Egypt, coyotes in the USA, have

all been suggested as the reservoirs of *B.gibsoni* (Maronpot and Guindy, 1970; Botros et al., 1975; Yamane et al., 1994). Roher et al. (1985) documented that when coyotes were experimentally infected with *B.gibsoni* exhibited only mild clinical signs, describing that they may act as carrier animals. Cape hunting dogs and silver-backed jackals have been associated with *B.canis* infection (Kuttler and Aliu, 1984). *Babesia canis rossi* was found in the blood of side striped jackals in Southern Africa (Lewis et al., 1996) and *T. anne* has been identified in red foxes in Spain (Criado-Fornelio et al., 2003a) and the USA (Goethert and Telford, 2003). Wild canines (dingoes) in Australia have been previously reported with babesiosis (Callow, 1984) and were probably infected with *B.canis vogeli* (Irwin and Hutchinson, 1991). In 1959, Abdulssalam reported the occurrence of *Babesia gibsoni* in dogs and jackals throughout Indo-Pakistan (cited by Bashir, 2008).

### 2.7 Life cycle

The life cycle of canine piroplasms is characteristic of that of all apicomplexan parasites in that it generally involves at least three phases of reproduction; gamogony, sporogony and schizogony (Homer *et al.,* 2000; Kjemtrup and Conrad, 2000). Schizogony occurs within the vertebrate host and the stages gamogany and sporogany occur within the tick vector. Detailed studies have determined many stages within the lifecycle of both *B. canis* and *B. gibsoni*, however no lifecycle stages have been determined for *Babesia* spp (North Carolina).

#### 2.7.1 Stages in the tick vector

The life cycle of *B. canis* is shown in Figure 5. Detailed observations of the development of *B. canis* within the gut of the adult tick *Dermacentor reticulatus* have been recorded (Mehlhorn and Schein, 1985). In addition, comprehensive studies have been carried out on the development of *B. gibsoni* within the midgut of both the larval and nymphal stages of the tick *R. sanguineus* (Higuchi et al., 1999). Development is similar for both *B. canis* and *B. gibsoni* and involves the sexual reproductive stage of the life cycle. Merozoites, and trophozoites within canine erythrocytes, are ingested by the tick vector and are microscopically detectable in the gut of the tick about 10 hours after feeding commences (Homer et al., 2000). The trophozoites develop into gametocytes and begin to form a ray body at the anterior of the piroplasm. These in turn form gametes and fuse to produce a zygote, which enters the gut epithelium cells.

At this stage, the zygote becomes a kinete which migrates to the salivary glands via the haemolymph (Mehlhorn and Schein, 1985). Kinetes can also enter the eggs of the tick, allowing for transovarial transmission (Homer et al., 2000). Sporogony or the formation of sporozoites occurs within the salivary gland, with many thousands of sporozoites being produced from each initial kinete.

### 2.7.2 Stages in the vertebrate host

Transmission of the sporozoites from the tick's salivary glands to the canine host generally occurs 2-3 days after tick attachment (Martinod et al., 1986). Once inside the host, the sporozoites become merozoites and invade the erythrocytes by a process of endocytosis and form a parasitophorus vacuole (which later disintegrates) within the cell (Homer et al., 2000). The merozoites transform into trophozoites and divide by binary fission into additional merozoites, a stage termed schizogony. The newly formed merozoites lyse the host cell and continue to invade and multiply within other erythrocytes. Some of the trophozoites become gametocytes, reproducing once inside of the tick gut.

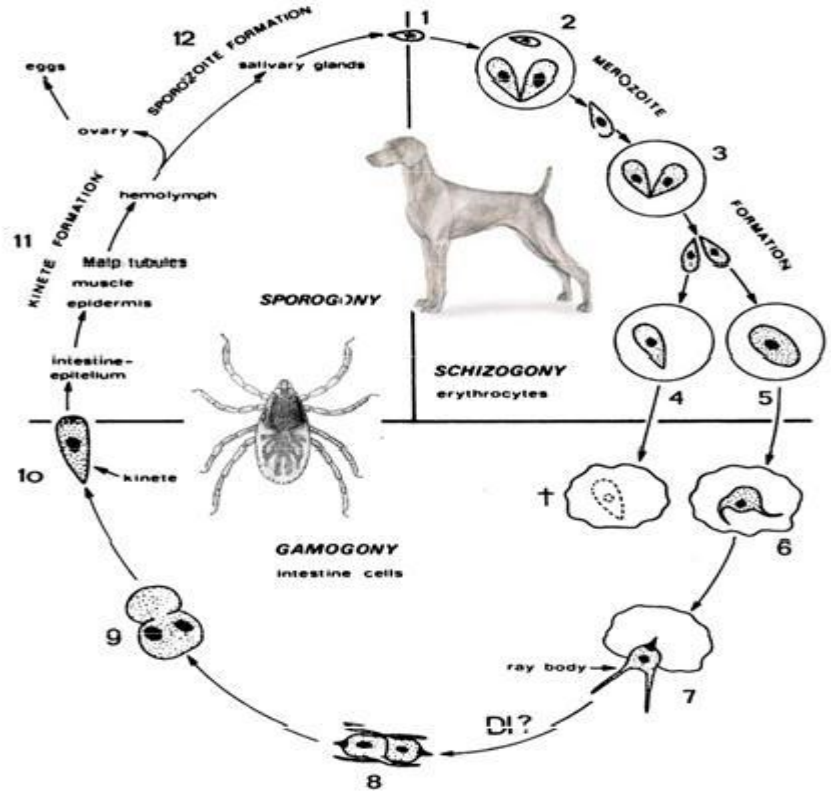


**Figure 5:** Typical three stage life cycle of *Babesia canis*.

1 to 5 – Schizogony within the canine host, 6 to 10 – Gamogany and 11 to 12 – Sporogany in the tick vector. **Modified from** (Mehlhorn and Schein, 1985).

## 2.8 Distribution

### 2.8.1 Large canine piroplasm species

The large canine piroplasm *Babesia canis vogeli* is distributed in tropical and sub-tropical areas of South and North America, Africa, Australia, Asia, Southern Europe and the Middle East (Uilenberg et al., 1989; Taboada et al., 1992; Carret et al., 1999; Cacciò et al., 2002; Jefferies et al., 2009). *Babesia canis rossi* is believed to have the most confined distribution, found only in Southern Africa (Uilenberg et al., 1989; Carret et al., 1999) and Sudan (Oyamada et al., 2005). *Babesia canis canis* has been reported in France, Hungry (Földvári et al., 2005), Neitherlands (Zandvliet et al., 2004), Solvenia (Duh et al., 2004), Russia (Rar et al., 2005), Switzerland (Casati et al., 2006) Poland and Croatia (Cacciò et al., 2002). The distribution of the unnamed large *Babesia* spp. is unknown and has only been found in one dog in North Carolina, USA (Birkenheuer et al., 2004).

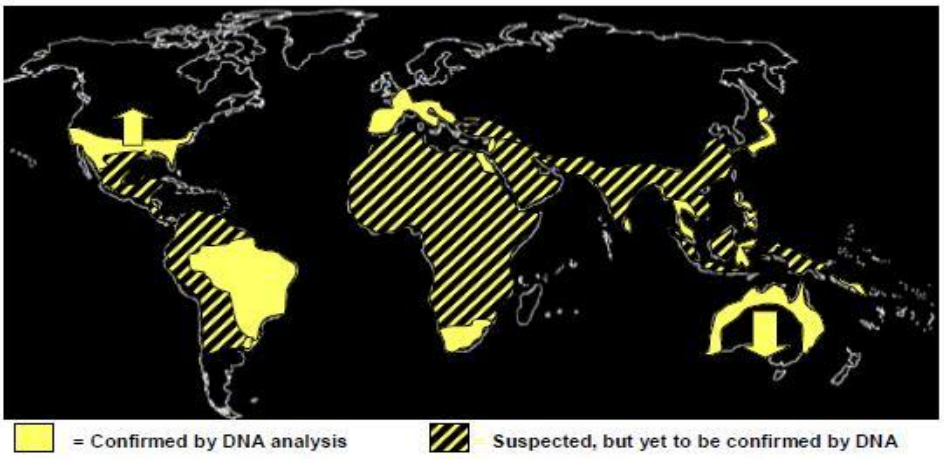


**Figure 6:** Worldwide distribution of *Babesia vogeli*

**Collected from** (Bashir, 2008)

### 2.8.2 Small canine piroplasm species

*Babesia gibsoni* is found in Bangladesh (Terao et al., 2015), India (Patton, 1910), Pakistan (Afzal et al., 1991), Japan, Malysia, Srilanka (Zahler et al., 2000b), Korea (Scott et al., 1971; Song et al., 2004), North America (Anderson et al., 1979; Birkenheuer et al., 1999), Italy (Casapulla et al., 1998), Spain (Criado-Fornelio et al., 2003b), France (Zahler et al., 2000a; Suarez et al., 2001), Egypt, Nigeria and Mali (Yamane et al., 1993b), and Australia (Muhlnickel et al., 2002).

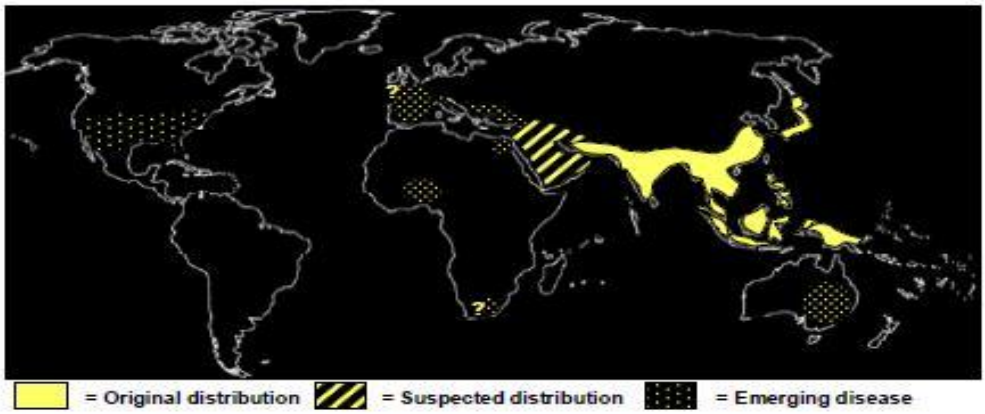


**Figure 7:** Worldwide distribution of *Babesia gibsoni*

**Collected from** (Bashir, 2008)

**Table 2:** Recent reports of canine blood sampling for the prevalence of *Babesia sp*

| Country | Sample size | Methods | Prevalence (%) | References |
|---|---|---|---|---|
| Bangladesh | 50 | PCR | 30% | (Terao et al., 2015) |
| | 272 | Case study | 1.64% | (Mahmud et al., 2014) |
| | 68 | PCR | 38.2% | (Talukder et al., 2013) |
| | 543 | Case study | 0.08% | (Tarafder and Samad, 2012) |
| India | 214 | Microscopic | 7.47% | (Singh et al., 2014) |
| | 532 | Microscopic | 5.82% | (Singh et al., 2012) |
| | 116 | Microscopic | 12.06% | (Polak et al., 2012) |
| | 261 | PCR | 5.5% | (Rani et al., 2011) |
| Pakistan | 5669 | Microscopic | 12.49% | (Ahmad et al., 2007) |
| Thailand | 303 | Microscopic | 13.2% | (Laummaunwai et al., 2014) |
| | | PCR | 19.5% | |
| Iran | 400 | Microscopic | 3.75% | (Jalali et al., 2013) |
| Japan | 196 | Microscopic | 1.6% | (El-Dakhly et al., 2014) |
| | | PCR | 4.1% | |
| Brazil | 129 | Indirect Immuno Flurosescence Assay | 42.6% | (Spolidorio et al., 2013) |
| America | 39 | Q-PCR | 46% | (Wei et al., 2014) |
| Great Britain | 742 | PCR | 2.4% | (Smith and Wall, 2013) |
| Nigeria | 108 | Microscopic | 10.2% | (Amuta et al., 2010) |
| Norway | 1908 | Real time-PCR | 0.9% | (Oines et al., 2012) |

## 2.9 Clinical signs and pathogenesis

*Babesia* spp. cause disease in the vertebrate hosts through a combination of both direct parasite induced damage and secondary immune mediated effects (Ristic et al., 1982; Homer et al., 2000). Clinical signs include pale mucous membranes, depression, anorexia and jaundice (Irwin and Hutchinson, 1991). Less virulent strains produce a more transient disease while those that exhibit an increased virulence can produce multiple organ dysfunctions, which can lead to the death of the infected host (Lobetti et al., 2002; Boozer and Macintire, 2003). Babesiosis can be classified as acute, chronic or subclinical (Breitschwerdt et al., 1999).

The recrudescence of infection is possible in Piroplasmida species (Bronsdon et al., 1999). Re-emergence of clinical infection in animals previously known to be infected with a pathogen is often induced by increased stress levels in the host. It highlights the possibility that Piroplasmida species can remain inactive within certain organ system, while not being present in the circulatory system (Ilhan et al., 1998). Studies show that inactive piroplasm may exist within the spleen, liver, kidneys or brain of the host, producing no illness for months and even years (Dao and Eberhard, 1996; O'Connor et al., 1999; Jefferies, 2006). Lobetti et al. (2002) documented that the acute phase of the infection of all three subspecies is characterized by haemolytic anemia, acute renal failure, cerebral babesiosis, coagulopathy, icterus, hepatopathy, immune mediated haemolytic anemia, acute respiratory syndrome and shock as complications associated with *B.canis* infection. Irwin and Hutchinson (1991) reported that each of *B.canis* subspecies produce different disease syndrome, which is further supported by (Schetters et al., 1995). Keller et al. (2004) suggested that hypoglycemia and icterus are also associated with *Babesia* infection.

## 2.10 Diagnosis

Accurate and correct diagnosis of babesial infections plays an important role in monitoring, management and control (McLaughlin et al., 1992). A large diversity of diagnostic techniques exist, each of which has its own limitations. The diagnostic tests for piroplasmosis can be divided into three categories; traditional methods, including microscopy and culture; serological techniques; and molecular based methods. It is increasingly recognized that a combination of detection techniques is necessary for accurate diagnosis. Limitations of clinical data, parasite morphology and serological

cross-reactivity, have lead to an increased interest in molecular based methods and highlights the need for their application in clinical medicine (Jefferies, 2006).

### 2.10.1 Light microscopy

Microscopy was the first technique utilized to identify piroplasms and remains the most common diagnostic technique for researchers and clinicians for the diagnosis of babesiosis. Thin blood smears stained with Wright or Geimsa stain are examined for the presence of the protozoans (Homer et al., 2000). Greene (2013) documented that the level of parasitemia can be calculated in blood smears and blood taken from ear tip capillaries increase the chances of detecting piroplasm infections. (Böse et al., 1995), suggested sensitivity of microscopy to be one parasite per $10^5$ erythrocytes. This technique is limited in that morphologically similar species cannot be distinguished (Conrad et al., 1992) and accurate diagnosis is dependent on the experience of the microscopist (Morgan, 2000).

### 2.10.2 Serological tests

Many serological techniques have been created to detect antibodies to *Babesia* spp reviewed by (Böse et al., 1995).

### 2.10.3 Complement Fixation test (CFT)

Complement fixation tests were utilized for the diagnosis of bovine babesiosis, but have been replaced for more sensitive and specific tests (Todorovic and Long, 1976; Kuttler et al., 1977; Bidwell et al., 1978).

### 2.10.4 Immunofluorescent Antibody test (IFAT)

This test is a commonly used method of diagnosing *Babesia* and *Theleria* infections by detecting the presence of antibodies to the parasities within the host serum. The test uses antigen, in the form of parasite-infected blood applied to glass slides, host serum titrated to various dilutions and flourescein-labelled antibodies. The serum and antibodies are added to the antigen, incubated and analysed using fluorescent microscope (Jefferies, 2006).

Indirect fluorescent antibody testing to detect the presence of anti-*Babesia* antibodies is commonly used, but is plagued with a lack of sensitivity and specificity (Donnelly et al., 1980; Akinboade and Dipeolu, 1984; Shkap et al., 1998; Goff et al., 2003). It is

also reported that dogs that are acutely infected with *Babesia* may be seronegative (Greene, 2013) and it is also difficult to assess whether the dog currently has an infection or has previously been infected. Seroreactivity against *Babesia* antigens does not definitively prove that an animal is actively infected with *Babesia,* and there are several reports of seronegative animals that were confirmed to be infected by microscopy and or PCR.

### 2.10.5 Enzyme Linked Immunosorbent Assay (ELISA)

Other antibody detection based methods, such as enzyme linked immunosorbant assays (ELISA), have been described, but few are commercially available (Voller et al., 1976; Jidaiso, 1986; Böse et al., 1990; Doherr, 1990; Makimura et al., 1990; Böse and Peymann, 1994). Martinod et al. (1986) used ELISA to detect canine babesiosis. The study developed the assay to detect antibodies against *B.canis* and also to the antibodies to the vectors. The ELISA is limited by *Babesia* strain differences eliciting different antibody responses and producing variable seroreactivity (Reiter and Weiland, 1989).

### 2.10.6 Animal inoculation test

Sensitive and specific tests such as sub-innoculation of blood into rodents or susceptible splenectomized animals have been used in human medicine and research settings, but are impractical for routine diagnostic purposes. The inoculation of laboratory animals with blood from presumed cases of babesiosis is time consuming because the first symptoms occur after 10 days (Skotarczak, 2008).

### 2.10.7 Polymerase Chain Reaction (PCR)

One of the most frequently used tools in the detection of *Babesia* protozoans is PCR. The selection of appropriate genetic markers to detect *Babesia* DNA is extremely important (Skotarczak, 2008). The first PCR test for *Babesia* was described by (Fahrimal et al., 1992). Since that time, a number of PCR based tests have been developed (Figueroa et al., 1992; Persing et al., 1992; Bashiruddin et al., 1999; Salem et al., 1999; Birkenheuer et al., 2003a; García, 2006; Bashir, 2008; Kamani et al., 2010). The majority of these tests have been based on 18S rRNA gene sequences, but other genes such as the internal transcribed spacer region, cytochrome b, and P50 have also been used as gene targets. Studies comparing the sensitivity of PCR tests for

diagnosis of babesiosis to detection by microscopy have found that PCR is more sensitive in most cases (Almeria et al., 2002). PCR is highly sensitive and specific (Laummaunwai et al., 2014). Primers used in the test can be designed to be genus specific or can amplify species-specific sequences of DNA, so can detect a single species. (Ano et al., 2001; Jefferies, 2001; Birkenheuer et al., 2003a) all assessed the PCR sensitivity for detecting canine *Babesia* by serially diluting blood samples of a known percentage parasitaemia (ranging from 0.000118- 0.00000073%). The high degree of sensitivity of PCR is important in diagnosing acute infections when the parasitaemia is low (i.e <1%). (Krause et al., 1996) documented that PCR is more sensitive than blood smear examination and IFAT for the diagnosis of acute *Babesia* infections.

#### 2.10.7.1 PCR detection of Babesia DNA in canine blood

Allsopp et al. (1994) demonstrated the PCR application to canine *Babesia*, involving DNA amplification for sequencing and phylogenetic comparison. Later studies have shown the ability of PCR to be a useful diagnostic tool for the detection and phylogenetic analysis of the canine *Babesia.* Carret et al. (1999) documented that the amplification of partial region of the small subunit ribosomal RNA gene is the basis of diagnosis. The small subunit ribosomal RNA gene is useful as they are highly conserved gene, showing limited nucleotide sequence variation. The gene exhibits a steady accumulation of mutations on an evolutionary scale and is therefore valuable in distinguishing different species (Hillis and Dixon, 1991). Different regions of the small subunit ribosomal RNA gene have been amplified by PCR, including the 18S rRNA gene (Conrad et al., 1992; Kjemtrup et al., 2002; Birkenheuer et al., 2003a) the first and second transcribed spacers (ITSI and ITS2) and the 5.8S rRNA gene for *B.canis* (Zahler et al., 1998).

#### 2.10.7.2 PCR detection of *Babesia* DNA in ticks

Sparagano et al. (1999) reviewed that PCR has also been applied to the detection of pathogen DNA within tick vectors and documented that the main problem associated with PCR analysis on ticks is contamination by non-target organisms on the surface of the ticks, which can be overcome by ethanol sterilization.

### 2.10.7.3 Nested and semi-nested PCR

Ano et al. (2001) tested the sensitivity of the nested PCR protocol carried out on the basis of the gene fragment encoding 18S rRNA in dogs experimentally infected and in naturally infected patients. They found that visualization of the product after the first-round of PCR was poor in both groups and that only after the second round was there a clear band on the agarose gel.

With the aim of developing the molecular diagnostics of babesiosis, Birkenheuer et al. (2003a) carried out semi-nested PCR to detect and differentiate the DNA of *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *Canis*, and *B. canis* subsp. *rossi* in canine blood samples in the USA. They designed pairs of primers to amplify an approximately 340-bp fragment of the 18S rRNA genes from *B. gibsoni* (Asian genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *rossi*, and *B. canis* subsp. *canis* but not mammalian DNA. The authors emphasized that in the diagnostics of babesiosis, the determination of the species, subspecies and even genotype that caused the babesiosis in dogs is very essential, because virulence, prognosis and response to medicines against *Babesia* may be different in every organism.

### 2.10.7.4 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Zahler et al. (1998) reported that RFLP use allows the discrimination of amplified DNA products on the basis of nucleotide differences. Restriction enzymes are used to cleave DNA at specific sites, so as to produce different range of smaller DNA fragments which thus can be used as a means of differentiating species or genotypes. Each of the *B.canis* subspecies has also been differentiated by RFLP using a partial region of the 18S rRNA gene (Carret et al., 1999). This method reduces the time and cost of detection and differentiation as the amplified DNA does not need to be sequenced.

### 2.10.7.5 Quantitative PCR (Q-PCR)

Quantative PCR or Q-PCR enables the estimation of the initial concentration of target DNA within a sample using various fluorescence technologies. Jeong et al. (2003) developed Q-PCR for the detection and quantification of *Theileria sergenti* using Taq Man chemistry. The TaqMan Q-PCR was reported to detect parasitaemia of 0.00005%, making it highly useful in detecting chronic infection and also in effective

determination of parasitaemia status in cattle. It has not yet been reported for the detection of the canine piroplasms.

### 2.10.8 Loop-mediated Isothermal Amplification method (LAMP)

Notomi et al. (2000) described first time the loop-mediated isothermal amplification method which permits under isothermal conditions the amplification of DNA with high level of specificity, efficiency and rapidity. Four primers were used that recognize six distinct regions on the target DNA and DNA polymerase, and as a result multiple stem-loop DNA structures are synthesized. The cycling reaction can produce 109 copies of the target region of the DNA in less than an hour time (Notomi et al., 2000). Ikadai et al. (2004) developed a LAMP assay for the detection of *B.gibsoni*. The LAMP reaction requires one hour time limit whereas, PCR can take up to four hours (Ikadai et al., 2004).

### 2.10.9 Filter Paper-based DNA detection

Belgrader et al. (1995) developed the use of filter paper for the storage and archiving of DNA samples for further amplification. A number of commercial filter papers including Isocode Stix and Whatman FTA cards were later developed as a method of collection, shipment, archiving and purification of DNA from blood and tissue samples for PCR analysis. FTA treated papers contains protein denaturants, chelating agents and a free radical trap designed to enable the protection and long term binding of the DNA to the filter matrix (Belgrader et al., 1995). Other substances within the sample, such as potential PCR inhibitors found in blood, are not bound to the FTA matrix and can be removed during serial washing of the sample. Li et al. (2004) reported that the samples stored on FTA cards show significant archiving potential, with DNA stability shown to exist after greater than four years. The use of filter-based technology has primarily been for forensic applications whereby DNA can be isolated directly from mammalian and plant tissues (Natarajan et al., 2000; Raina and Dogra, 2002). This technique has been used for the detection of *B.microti* DNA (Okabayashi et al., 2002) and for canine piroplasm (Jefferies, 2006).

### 2.10.10 Other methods of detection

Other methods of detection include the hydroethidine-flow cytometry method (Bicalho et al., 2004), reverse line blot hybridization assays (Gubbels et al., 1999;

Georges et al., 2001; Almeria et al., 2002) and the latex agglutination test (Xuan et al., 2001).

### 2.11 Prevention of piroplasms infections

The most successful for the prevention of babesiosis is to avoid exposure to ticks (Smith and Kakoma, 1989; Homer et al., 2000). Transmission of parasite can also be limited by the removal of ticks within 24 hours of attachment, as there is direct correlation between attachment time and the transmission of sporozoites (Homer et al., 2000).

Pre-immunization is referred to as the immunity of infection, and is a state in which the animal remains infected, but has very few clinical signs. In many countries where re-infection is nearly impossible to prevent, the goal is to induce a state of pre-immunization through drug treatments that reduce the severity of clinical signs, but do not result in complete sterilization of the *Babesia* infection (Kuttler and Aliu, 1984; Penzhorn et al., 1995). This strategy has been used in some areas, where animals are purposely infected with a mildly pathogenic strain of the organism (i.e. live vaccine) to achieve protection from severe clinical disease when the animal is exposed to more pathogenic field strains. Dogs that are initially infected with *Babesia* often do not become re-infected due to the effect of protective immunity. In case of *Babesia canis* infection, immunity existed for at least 5 months (and even up to 8 months) (Vercammen et al., 1997). Schetters et al. (1997) and Vercammen et al. (1997) reported that there is no cross protection between the subspecies of *B. canis* it gives the idea of antigenic variation exists between the species. Many vaccines based on soluble parasite antigens have been developed for *B. canis* infections and some are available commercially (Moreau et al., 1989; Schetters et al., 1995; Schetters et al., 1997). Whereas Fukumoto et al. (2000) suggested the use of recombinant surface antigen P50 for immunization against *B. gibsoni*.

# Chapter III: Materials and methods

## 3.1 Description of study areas

The study was conducted at Chittagong Metropolitan area (CMA) of Bangladesh. Among the total CMA, nine locations were randomly selected for the sampling such as Panchlaish, Chawkbazar, Muradpur, Alankar, Kattoli, Pahartali, Bayzid, Ambagan and New market. The locations were chosen on the basis of high density of stray dog in CMA (Das et al., 2012). Moreover, selection of study areas were based on probability of high prevalence of different vectors, the climatic condition and geographical location which might favors the occurrence of such diseases.

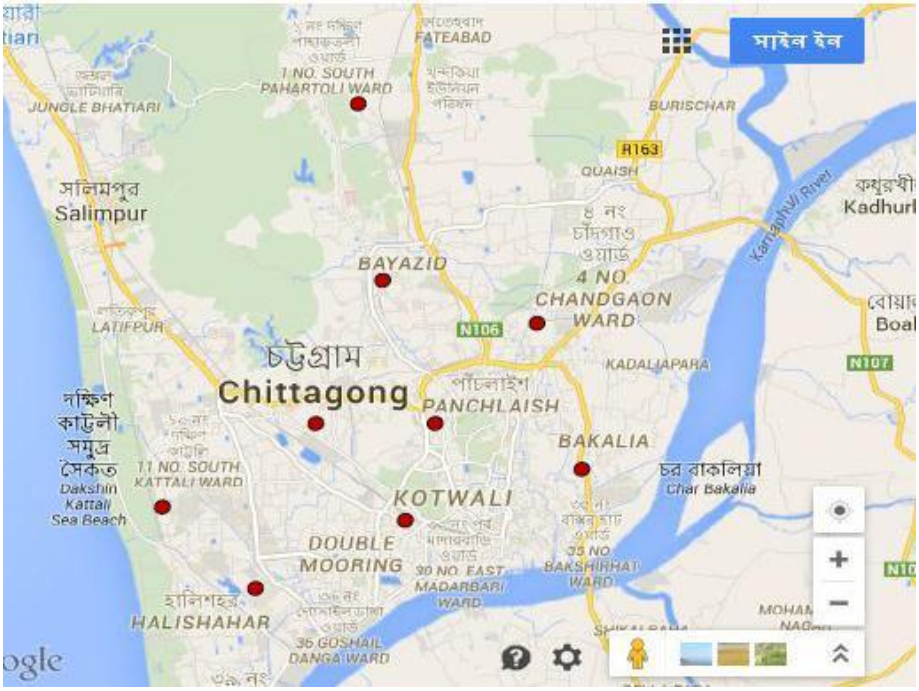


**Figure 8:** Study area. (Chittagong Metropolitan area)

## 3.2 Study period

The study was undertaken for a period of 6 months from July 2014 to December 2014.

## 3.3 Selection of animals and Survey Design

The survey was conducted as cross sectional study.

### 3.4 Target animals and age groups

Stray dogs from the Chittagong Metropolitan area were selected for this study as target sample. To determine the age susceptibility to babesiosis the stray dogs were categorized as young (below one year) and adult (above one year). The approximate age of the stray dogs was estimated by examining the teeth. Dogs having all white and shiny permanent teeth without worn off cups on the incisors were considered as young (below one year) and dogs having teeth yellowish discoloration and tarter formation with worn cups on the incisors were considered as (above one year) adult (Cynthia et al., 2011).

### 3.5 Target sampling

A total of 130 blood samples were collected randomly from stray dogs of Chittagong Metropolitan area. A prototype questionnaire was used to record the information like area, age, sex, etc.

### 3.6 Dog catching and handling for sample collection

The process of dog handling and catching was done by humane method (also known as 'ethological' handling). The process is defined as causing the minimum amount of stress possible during the procedure to both the animal and the people involved (FAO, 2014). In order to achieve humane handling, the individual dog's behaviour and the immediate environment was taken into account.

### 3.7 Sample collection and preservation

Only one biological sample (blood) was collected during this study where an individual animal was considered as a sampling unit. Smears were prepared form blood obtained from ear vein puncturing with sterile needle. Two thin blood smears were prepared by touching the coming out fresh blood and then spread by another slide. The slides were air dried and fixed by 100% methyl alcohol for 3-5 minutes (Cable, 1950). After making the slide the remaining portion of blood were kept into -20° C.

### 3.8 Examination of samples

All the examinations were carried out at the Parasitology laboratory, Chittagong Veterinary and Animal Sciences University (CVASU). The prepared thin blood smears (Hendrix and Robinson, 2006) were stained with the Giemsa stain for 25-30 minutes. After rinsing with water, the stained blood smears were air dried and examined under binocular microscope (X100) with immersion oil for the identification of blood parasites (Soulsby, 1982; Sloss et al., 1994; Urquhart et al., 1996).

### 3.9 Protocol used for DNA isolation from blood sample

Total genomic deoxyribonucleic acid (DNA) has been extracted from the whole blood samples by using PCI method (Sambrook et al., 1989).

- Blood samples typically were obtained as 1 ml of whole blood stored in EDTA vacutainer tubes which frozen at 4°C.
- The Blood sample was pipetted in a sterilized microcentrifuge tube containing 500 µl of Lysis buffer.
- To the above sample, added 50µl of 10% SDS and mixed thoroughly for few minutes.
- Added 2-3 µl of Proteinase-K.
- Mixed to the sample thoroughly by inverting the micocentrifuge tube for minutes.
- It is then incubated at 56°C for 30 minutes.
- Added equal volume of phenol: chloroform: Isoamylalcohol (25:24:1).
- Mixed thoroughly for few minutes.
- Centrifuged the sample for 10 minutes at 13,000 rpm centrifuge machine.
- Carefully removed the aqueous layer to a new sterilized microcentrifuge tube.
- Added 500 ml of Chloroform: Isoamyl alcohol (24:1).
- Mixed thoroughly by repeated inverting the microcentrifuge tube for few minutes.
- Centrifuged at 13,000 rpm for 10 minutes in a centrifuge machine.

- Transferred the upper aqueous layer in a fresh sterilized microcentrifuge tube and added double the volume of chilled absolute ethanol.
- The above sample was kept at -20°C for overnight for precipitation.
- The above sample was centrifuged at 10,000 rpm for 10 minutes.
- Decanted the supernatant and retained the pellet.
- To the pellet, added 500 µl of 70% ethanol and again centrifuged at 13,000 rpm for 10 minutes and decanted the supernatant in -4°C.
- The pellet was kept for air dry under laminar air flow.
- The pellet was resuspended in Nuclease free water stored either in -20°C or -86°C for immediate use or long preservation.

DNA extracted from the blood samples were processed for PCR detection and confirmation of the disease. All laboratory works were undertaken in Molecular biology laboratory of Poultry Research and Training Centre (PRTC), CVASU.

### 3.10 Confirmative diagnosis of disease by PCR

Ultimate confirmatory diagnosis was made on the basis of the PCR and DNA sequences. Although there were different types of specific techniques the study aims to impose on the commonly used rapid diagnostic tools beside of molecular technique (PCR and Real-time PCR). Again, the study also considers only those tools which are more commonly used for wide range of organism to diagnose.

### 3.11 *Babesia* spp identification by PCR

The extracted DNA from all microscopic positive samples of dogs' blood were subjected to PCR by using single set of primers containing part of 18S rRNA gene sequence. It was amplified and 422–440bp for *Babesia* spp (Hilpertshauser et al., 2006). After amplification the PCR, mixture was subjected to 1% agarose gel electrophoresis.

**Table 3:** Details of the primers used for PCR

| PCR primer | Primer name | Sequences (5″-3″) | Size (bp) |
|---|---|---|---|
| Babesiosis | bab- F | 5GTTTCTGMCCCATCAGCTTGAC-3’ | 420-440 |
| | bab- R | 5CAAGACAAAAGTCTGCTTGAAAC-3 | |

**Table 4:** PCR Master mix solution

| | |
|---|---|
| 2x PCR Master mix solution (i-Taq$^{TM}$) | 0.5 ml x 2 |
| Taq$^{TM}$ DNA polymerase (5U/µl) | 2.5 U |
| dNTPs | 2.5 mM each |
| PCR reaction buffer | 1X |
| Gel loading buffer | 1X |

[Catalog no. 25027 (1ml); 25028 (5ml); Intron Biotechnology] used for PCR contained the following reagents

**Table 5:** Composition of reaction mixture for PCR

| SL No. | Components | Quantity | Total amount | Final Concentration (20 µl) |
|---|---|---|---|---|
| 1 | 2X PCR master mix | 10 µl | 10 µl | -- |
| 2 | Forward Primer (10 pmole/µl) | 1µl | 1 µl | 10 pmole |
| 3 | Reverse Primer (10 pmole/µl) | 1 µl | 1 µl | 10 pmole |
| 4 | Water | 6 µl | 6 µl | -- |
| Total volume | | 18 µl | 18 µl | -- |
| 5 | DNA Template | 2 µl | 2 µl | -- |
| Grand Total volume | | 20 µl | 20 µl | -- |

### 3.12 PCR detection assay

To detect the *Babesia* from samples PCR amplifications were performed at the following thermal conditions: 94°C for 2 min followed by 45 cycles of 94°C for 30 sec, 61°C for 45 sec, 72°C for 1 min and followed the final extension step at 72°C for 10 min (Hilpertshauser et al., 2006).

**Table 6:** Steps and conditions of thermal cycling for *Babesia* spp.

| Sl No. | Steps | Temperature | Time |
|---|---|---|---|
| Step 1 | Initial Denaturation | 94°c | 2 min |
| Step 2 | Denaturation | 94°C | 30sec |
| Step 3 | Annealing | 61 °C | 45 sec |
| Step 4 | Extension | 72 °C | 1min |
| Step 5 | 45 cycles from step 2 to step 4 | | |
| Step 6 | Final Extension | 72 °C | 10 min |

After completing the PCR, the tubes containing PCR products were removed from the thermo cycler and stored at 4°C in a refrigerator until electrophoresis.

### 3.13 *Babesia sp.* and sub-species identification by PCR

It is diagnostically important to determine the species, sub-species and genotype that cause canine babesiosis. For definitive diagnosis of canine babesiosis, as well as the differentiation of the species of piroplasms, a seminested PCR was performed.

#### 3.13.1 Primer design

For the seminested PCR, an outer primer pair (455-479F and 793-772R) was designed that would amplify an approximately ~340 bp for *Bebesia gibsoni* (Asian type) (AF271081, AF27182), *Babesia canis*, *B. canis* subsp. *canis* (AJ009795), *B. canis* subsp. *vogeli* (AJ009796) and *B. canis* subsp. *rossi* (L19079) (Birkenheuer et al., 2003a).

Then specific internal primers were designed for *B. gibsoni* (Asian type) (BgibAsia-F) and *B. canis* subsp. *canis* (BCC-F) that were paired with the outer reverse primer

in the seminested secondary reaction to amplify 185 bp and 198 bp respectively (Birkenheuer et al., 2003a).

**Table 7:** Sequences for the oligonucleotide primers used in this seminested PCR

| Primer | Sequence (5′- 3′) | Reaction and /or use |
|---|---|---|
| 455-479F | GTCTTGTAATTGGAATGATGGTGAC | Seminested PCR outer forward primer |
| 793-772R | ATGCCCCCAACCGTTCCTATTA | Seminested PCR outer reverse primer |
| BgibAsia-F | ACTCGGCTACTTGCCTTGTC | Seminested PCR *B. gibsoni* (Asian type) forward primer |
| BCC-F | TGCGTTGACGGTTTGACC | Seminested PCR *B. canis* subsp. *canis* forward primer |

**Table 8:** Composition of reaction mixture for primary nested PCR

| SL No. | Components | Quantity | Total amount | Final Concentration (20 µl) |
|---|---|---|---|---|
| 1 | 2X PCR master mix | 10 µl | 10 µl | -- |
| 2 | Forward Primer (10 pmole/µl) | 1µl | 1 µl | 10 pmole |
| 3 | Reverse Primer (10 pmole/µl) | 1 µl | 1 µl | 10 pmole |
| 4 | Water | 7 µl | 7 µl | -- |
| Total volume | | 19 µl | 19 µl | -- |
| 5 | DNA Template | 1 µl | 1 µl | -- |
| Grand Total volume | | 20 µl | 20 µl | -- |

**Table 9:** Composition of reaction mixture for secondary nested PCR

| SL No. | Components | Quantity | Total amount | Final Concentration (20 µl) |
|---|---|---|---|---|
| 1 | 2X PCR master mix | 10 µl | 10 µl | -- |
| 2 | Forward Primer (10 pmole/µl) | 1µl | 1 µl | 10 pmole |
| 3 | Reverse Primer (10 pmole/µl) | 1 µl | 1 µl | 10 pmole |
| 4 | Water | 7 µl | 7 µl | -- |
| Total volume | | 19 µl | 19 µl | -- |
| 5 | DNA Template (PCR product of primary round ) | 1 µl | 1 µl | -- |
| Grand Total volume | | 20 µl | 20 µl | -- |

### 3.14 Nested PCR detection assay

#### 3.14.1 Primary reaction

To detect the *Babesia sp* from samples PCR amplifications were performed at the following thermal conditions: 95°C for 5 min followed by 50 cycles of 95°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec and followed the final extension step at 72°C for 5 min (Birkenheuer et al., 2003a).

**Table 10:** Steps and conditions of thermal cycling for *Babesia sp*

| Sl No. | Steps | Temperature | Time |
|---|---|---|---|
| Step 1 | Initial Denaturation | 95°c | 5 min |
| Step 2 | Denaturation | 95°C | 45 sec |
| Step 3 | Annealing | 58 °C | 45 sec |
| Step 4 | Extension | 72 °C | 45 sec |
| Step 5 | 50 cycles from step 2 to step 4 | | |
| Step 6 | Final Extension | 72 °C | 5 min |

### 3.14.2 Secondary reaction

To detect the *B. gibsoni* (Asian type) (BgibAsia-F) and *B. canis* subsp. *canis* (BCC-F) from samples PCR amplifications were performed at the following thermal conditions: 95°C for 5 min followed by 30 cycles of 95°C for 45 sec, 58°C for 45 sec, 72°C for 45 sec and followed the final extension step at 72°C for 5 min (Birkenheuer et al., 2003a).

**Table 11:** Steps and conditions of thermal cycling for *B. gibsoni* (Asian type) (BgibAsia-F) and *B. canis* subsp. *canis* (BCC-F)

| Sl No. | Steps | Temperature | Time |
|---|---|---|---|
| Step 1 | Initial Denaturation | 95°c | 5 min |
| Step 2 | Denaturation | 95°C | 45 sec |
| Step 3 | Annealing | 58 °C | 45 sec |
| Step 4 | Extension | 72 °C | 45 sec |
| Step 5 | 30 cycles from step 2 to step 4 | | |
| Step 6 | Final Extension | 72 °C | 5 min |

### 3.15 Agar gel electrophoresis

1% agarose gel was made by using 0.5 g agarose powder and 50ml TAE buffer with ethidium bromide. The DNA amplicons were visualized using 4 µl of the final PCR product and 2 µl standard 100bp DNA markers (Invitrogen) at 120 V/100mA for 30 min. Gels were photographed using a gel documentation system Positive or negative amplifications were evaluated as presence or absence of visible bands on agarose gels under UV light (O'Dwyer et al., 2009).

Briefly the procedure was as follows:

- For 1% agarose , 500 mg of agarose and 50 ml of 1 X TAE buffer was mixed thoroughly in a conical flask.
- The mixture was heated in a microwave oven until agarose was completely dissolved.
- The agarose-TAE buffer solution was then allowed to cool in room temperature.

- Gel casting tray was prepared by sealing ends of gel chamber with tape or appropriate casting system and placed appropriate number of combs in gel tray.
- 10 microlitre of ethidium bromide was added to agarose-TAE buffer mixture, shaked well and poured into gel tray.
- The gel was then allowed to be cool (left for 15-30 minutes at room temperature).
- The combs were removed and the electrophoresis chamber was filled with 1x TAE buffer untill the casted gel is drowned completely.
- 4 µl of DNA and 2 µl of 100 bp marker (ladder) were loaded into gel.
- The electrophoresis was run at 120 volt and 100 mA for 40 minutes.
- Then the gel were taken to the UV transilluminator for image acquisition and analysis.

### 3.16 PCR product clean up (FavorPrep™ DNA purification Mini Kit) and sequencing

The PCR products of representative strains were purified with the PCR Clean-up system kit and this products were sequenced at ICCDR,B (Bangladesh) where commercial sequencing is routinely performed.

### 3.17 Analysis of sequence chromatographs

The sequenced product was analyzed using the program (Chromas Lite version 2.1, 2012, Technelysium Pty Ltd, South Brisbane, Queensland, Australia) and was compared to sequence data available from GenBank™, using the BLAST 2.1 program. (http://www.ncbi.nlm.nih.gov/BLAST/).

### 3.18 Precautions followed in the PCR laboratory

All procedures were carried out under strict aseptic condition. Maximum precautions were taken to avoid contamination. Hand gloves were used all time, nothing were touched in bare hands to avoid contamination. All the samples were processed under the Biosafety cabinet Class II. Isolation of DNA, preparation of PCR reaction mix, thermo cycling and analysis were performed in three separate rooms to avoid carry-

over contamination. Eye protector was used while working with transilluminator (produces UV radiation) for documentation of PCR products.

### 3.19 Statistical Analysis

The obtained information was imported, stored and coded accordingly using Microsoft Excel-2007 to STATA/IC-11.0 (Stata Corporation College Station) for analysis. The result were expressed in percentage with P-value for Chi-Square Test. Significance was determined when $P<0.05$.

# Chapter IV: Results

## 4.1 Prevalence of the disease

All the samples were screened by microscope for identification of piroplasms in Giemsa's stain technique. Microscopic examination found a total of 9 positive (6.92%) samples. The piroplasms were seen as pyriform (pear) shape inside the red blood cells (RBC) (Figure: 9).

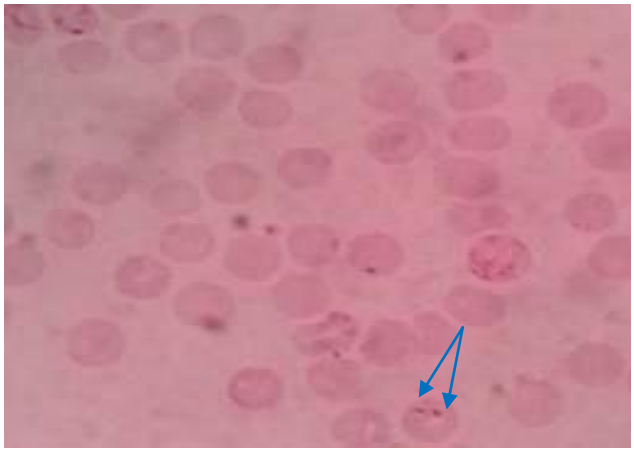

**Figure 9:** Pear shaped piroplasm inside RBC (×100).

Again the microscopy positive nine (9) samples were run by PCR using 18S rRNA gene and found 6 positive (66.67%) samples (Figure: 10).

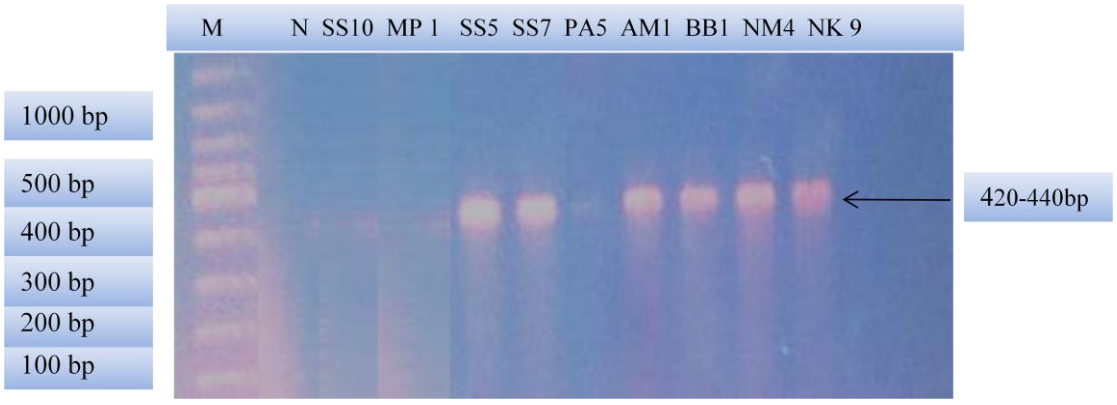


**Figure 10:** PCR products amplified using bab- F & bab-R specific primers.
Lane M: 100 bp DNA ladder (Invitrogen), lane N: negative control, lane 2, 3& 6: negative samples, lane 4, 5, 7, 8 & 9: positive samples.

**Table 12:** Overall prevalence and percentage of *Babesia* spp in microscopic and PCR techniques respectively

| Techniques | Total no. of sample (n=130) | |
|---|---|---|
| | No. of positive sample | Prevalence (%) |
| Microscopic | 9 | 6.92 |
| | Total no. of sample (n=9) | |
| | No. of positive sample | Percentage (%) |
| PCR | 6 | 66.67 |

**Table 13:** Relationship of age and sex to the prevalence of *Babesia* spp in stray dog

| Variables | | N | Microscopic examination | | | Confirmation of *Babesia* spp DNA by 18S rRNA gene PCR | | |
|---|---|---|---|---|---|---|---|---|
| | | | No. of positive | % | *P* value | Microscopic positive | PCR positive | *P* value |
| Age | Adult | 89 | 9 | 10.11 | 0.03* | 9 | 6 | No |
| | Young | 41 | 0 | 0.00 | | 0 | 0 | value |
| Sex | Male | 59 | 8 | 11.94 | 0.02* | 8 | 6 | 0.45 |
| | Female | 62 | 1 | 1.59 | | 1 | 0 | |

N= Total number, %= Percentage, *= $p<0.05$

Table (13) shows that the prevalence of *Babesia* spp in adult dog (10.11%) is higher than young and there is a significant difference between the two age groups in aspect of microscopic examination. Again, there is a significant difference within the two sex groups. Male dogs (11.94%) have more prevalence than female (1.59%).

**Table 14:** Prevalence of *Babesia* spp in different sampling site

| Variable | Categories | N | Prevalence (%) | $\chi^2$ value | *p* value |
|---|---|---|---|---|---|
| Sampling site | Muradpur | 29 | 13.79 | 6.00 | 0.65 |
| | Bayezid | 12 | 8.33 | | |
| | New market | 10 | - | | |
| | Panchlaish | 18 | - | | |
| | Chawkbazar | 18 | 5.56 | | |
| | Pahartali | 8 | - | | |
| | Alankar | 6 | 16.67 | | |
| | Kattoli | 19 | 5.26 | | |
| | Ambagan | 10 | 10.00 | | |

N= Total number, %= Percentage

Table (14) shows that, there was no statistically significant difference among the sampling sites for the prevalence of babesiosis in dog. The prevalence of babesiosis in stray dog was represented in (Figure: 11)

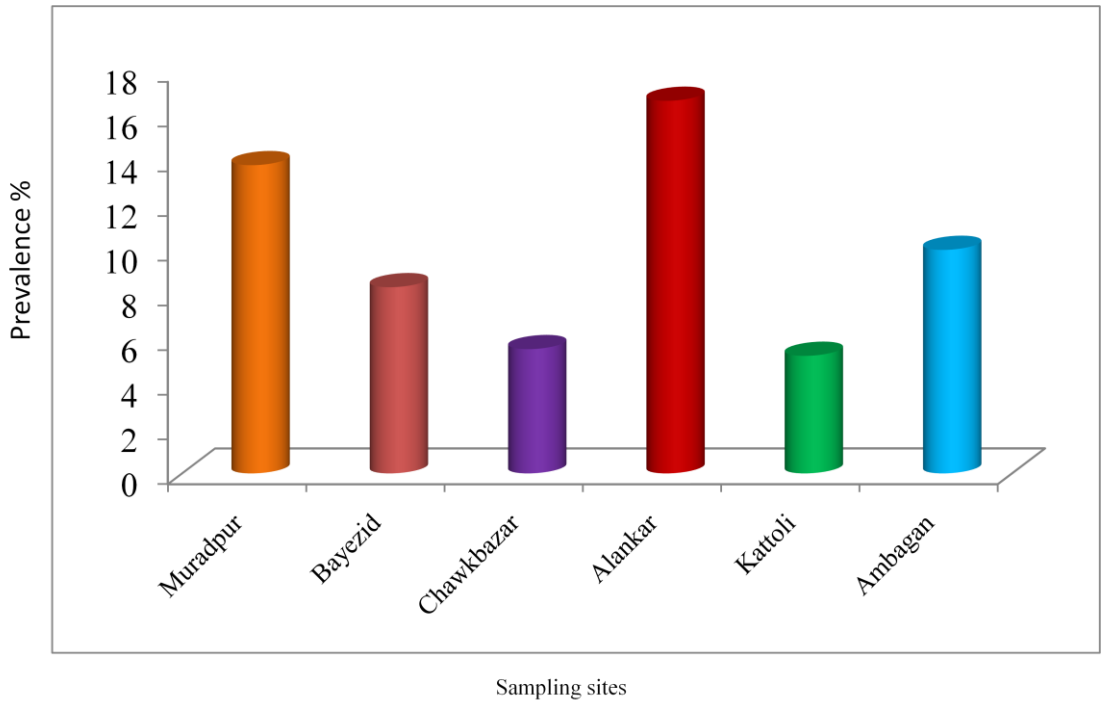


**Figure 11:** Prevalence of *Babesia* spp at different sampling sites

Fig (11) shows that, prevalence was highest (16.67%) in the samples of Alankar and lowest (5.26%) in the samples of Kattoli. But, there was no significant difference in the prevalence of babesiosis among the sampling sites.

### 4.2 Identification and characterization of *Babesia sp* by seminested PCR

*Babesia sp* was identified and characterized from the PCR positive samples run by 'Babesiosis primer' contained the sequences of 18S rRNA gene.

For species identification and characterization a seminested PCR performed. During the primary reaction of the seminested PCR, a ~340-bp product was amplified. This result confirmed that the positive samples belong to *Babesia gibsoni* (Asia genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis* or *B. canis* subsp. *rossi.*

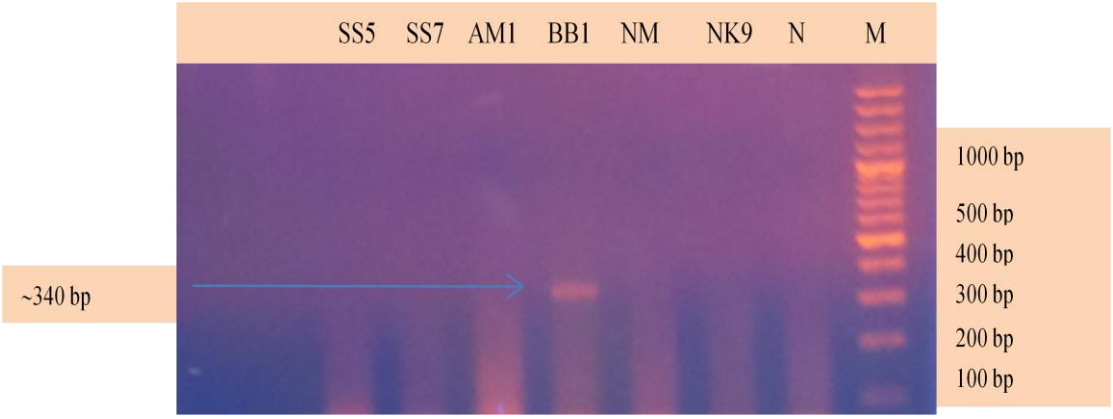


**Figure 12:** PCR products amplified using 455-479F & 793-772R specific primers.

Lane M: 100 bp DNA ladder (Invitrogen), lane N: negative control, lane 2, 3, 5, 6 & 7: negative samples, lane 4: positive sample.

During the secondary seminested reaction, the test was able to differentiate *Babesia gibsoni* (Asia genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis* and *B. canis* subsp. *rossi* when the specific internal primers were paired with the reverse primer. In the secondary seminested reaction I have used BgibAsia-F and BCC-F as internal primers and 793-772R as reverse primer. In the second round of seminested PCR, there were four (4) positive samples by the pairing of BCC-F and 793-772R (Figure: 13).

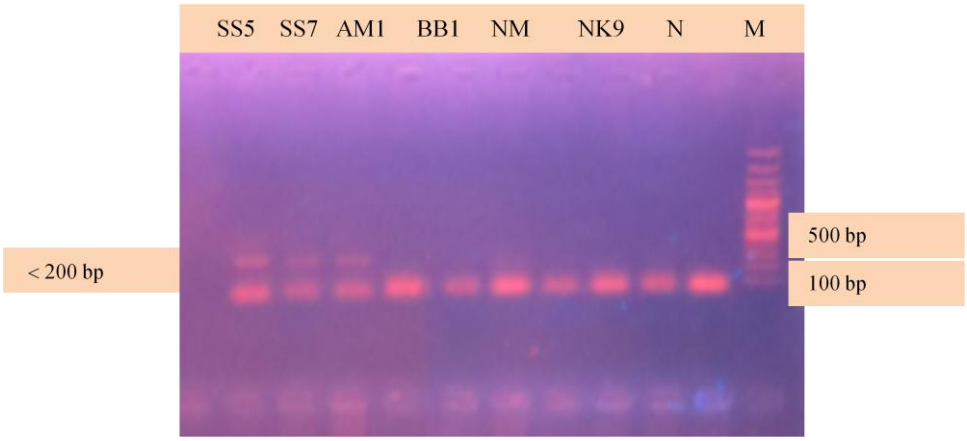


**Figure 13:** PCR products amplified using BCC-F & 793-772R specific primers.

Lane M: 100 bp DNA ladder (Invitrogen), lane N: negative control, lane 2, 3, 4, 5 & 6: negative samples, lane 7, 8, 9: positive sample.

Again, in the second round of seminested PCR, there were four (2) positive samples by the pairing of BgibAsia-F and 793-772R. Fig (14)

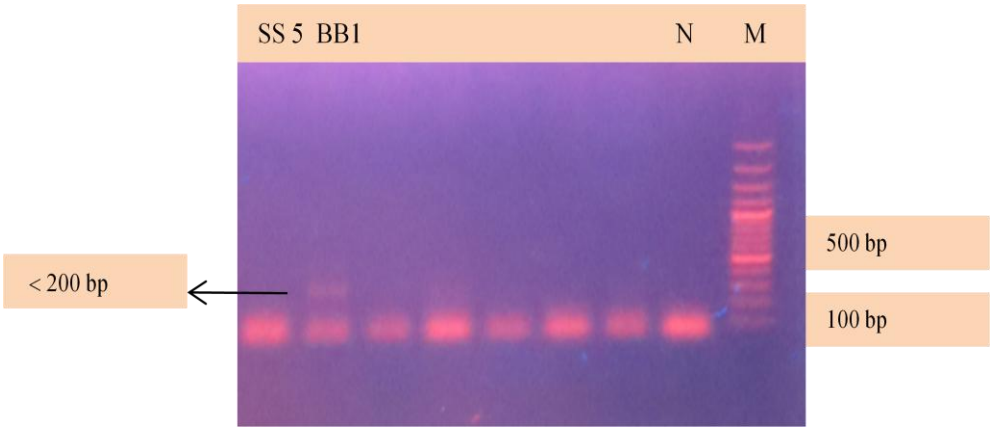


**Figure 14:** PCR products amplified using BgibAsia-F & 793-772R specific primers.

Lane M: 100 bp DNA ladder (Invitrogen), lane N: negative control, lane: 2, 3, 4, 5 & 7: negative samples, lane 6: positive sample.

### 4.3 Identification and characterization of *Babesia sp* by sequencing of PCR product

#### 4.3.1 Identification of nucleotide bases according to chromatogram peak

After sequencing of the representative PCR product, the quality of the sequence was assessed manually for each nucleotide. Fig (15) represents an example of partial chromatogram of sequence.

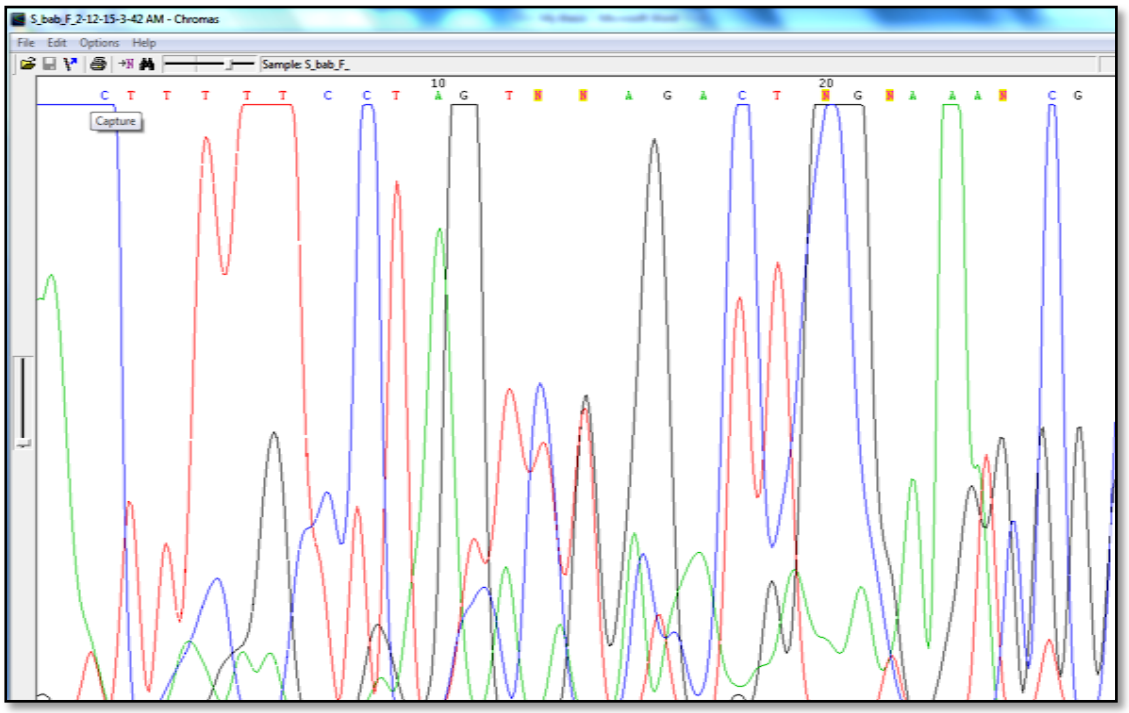


**Figure 15:** Partial chromatogram after sequencing of the PCR product.

**Where,** red, black, green and blue peaks indicate nucleotide bases T, G, A and C respectively.

#### 4.3.2 NCBI BLAST analysis

The sequence data was used to conduct non-redundant BLASTN analysis to characterize the *Babesia sp* and nucleotide sequence of the sample shown 95% identity with the (*Babesia gibsoni* gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: YGC15) (GenBank accession number LC012808.1). Fig (17)

Query ID: lcl|Query_50623

Description: S_bab_F_ sequence exported from S_bab_F_2-12-15-3-42 AM.ab1

Molecule type: Nucleic acid

Query Length: 359

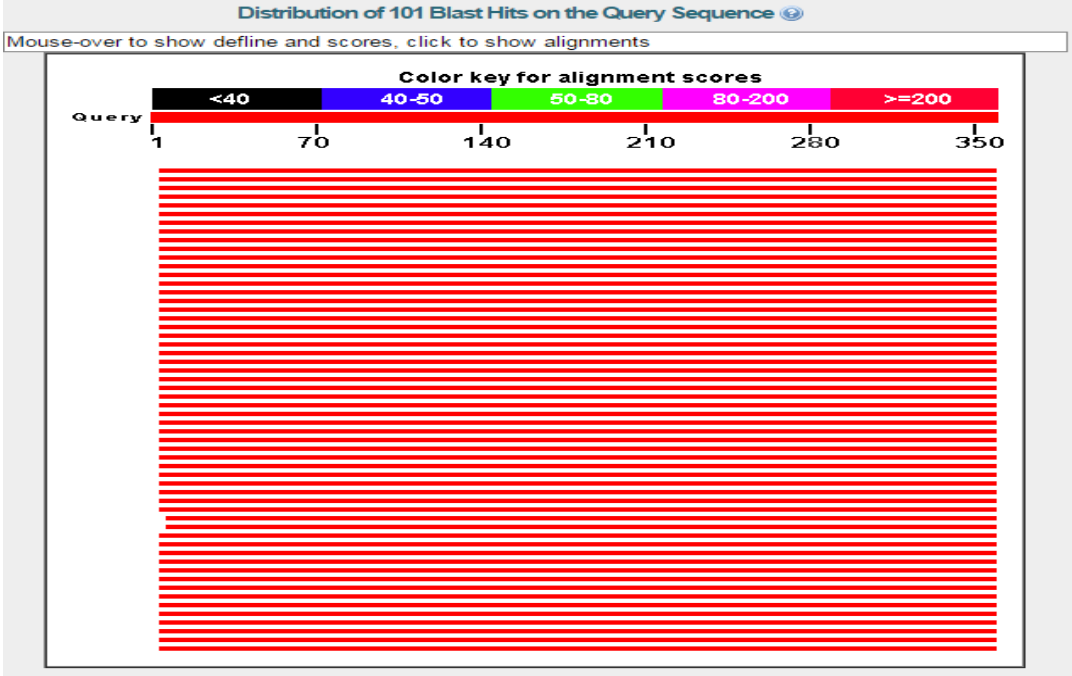


**Figure 16:** The graphic summary of 101 blast hits on the query sequence

**Sequences producing significant alignments:**

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

| Description | Max score | Total score | Query cover | E value | Ident | Accession |
|---|---|---|---|---|---|---|
| Babesia gibsoni gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: YGC15 | 518 | 518 | 98% | 8e-149 | 95% | LC012808.1 |
| Babesia gibsoni gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: OSK3 | 518 | 518 | 98% | 8e-149 | 95% | LC012799.1 |
| Babesia gibsoni gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: NGS6 | 518 | 518 | 98% | 8e-149 | 95% | LC012798.1 |
| Babesia gibsoni gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: KGS4 | 518 | 518 | 98% | 8e-149 | 95% | LC012793.1 |
| Babesia gibsoni gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: KGS1 | 518 | 518 | 98% | 8e-149 | 95% | LC012792.1 |
| Babesia gibsoni isolate Kolkata 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KJ142323.1 |
| Babesia gibsoni isolate CMVL-05/2013/Kiki 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF878947.1 |
| Babesia gibsoni isolate CMVL-03/2013/Mutant 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF878946.1 |
| Babesia gibsoni isolate CMVL-02/2013/Hasina 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF878945.1 |
| Babesia gibsoni isolate CMVL-04/2013/Bruty 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF878944.1 |
| Babesia gibsoni isolate CMVL-01/2013/Mahesh 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF878943.1 |
| Babesia gibsoni isolate Kolkata 2 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF171474.1 |
| Babesia gibsoni isolate Siliguri 18S ribosomal RNA gene, partial sequence | 518 | 518 | 98% | 8e-149 | 95% | KF171471.1 |

**Figure 17:** BLAST search across multiple DNA databases.

**By using** BLASTN software showed the homology of (*Babesia gibsoni* gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: YGC15) (GenBank accession number LC012808.1)

*Babesia gibsoni* gene for 18S ribosomal RNA, partial sequence, isolation source: blood of dog isolate: YGC15

Sequence ID: dbj|LC012808.1|Length: 1622Number of Matches: 1


**Figure 18:** The similarity of query sequence with GenBank.

Accession no. LC012808.1; Alignment shows 95 percent match between two sequences

### 4.3.3 Genbank accession number of submitted sequences

The sequence derived was later submitted to GenBank databases for accession number. Yet the sequence was not accepted by the GenBank.

# Chapter V: Discussion

## 5.1. Prevalence of babesiosis in stray dog

The overall prevalence of babesiosis in the current study by examination of Giemsa stained peripheral blood smears was higher than Singh et al. (2012), Jalali et al. (2013), El-Dakhly et al. (2014) where they found 5.83%, 3.75% and 1.6% in India, Iran and Japan respectively. The higher prevalence of the disease in the present study might be due to the variation of the area or availability of the vectors. Higher prevalence of the babesiosis in the study population suggested a continuous challenge of such infection in those areas.

Again the prevalence of the babesiosis in present study by microscopy was lower than Ahmad et al. (2007), Amuta et al. (2010), Polak et al. (2012), Laummaunwai et al. (2014), Singh et al. (2014) where they found 12.49%, 10.2%, 12.06%, 13.2% and 7.74% in Pakistan, Nigeria, India, Thailand and Northwest India respectively. The lower prevalence of babesiosis in the current study might be due to random sampling rather than selection of clinically susceptible dog. However, variation in geo-climatic condition and exposure of vectors might add to the erratic prevalence of blood parasitic diseases (Rahman, 2014).

Again the PCR percentage is higher in current study than the previous reports published in Bangladesh Talukder et al. (2013) and Terao et al. (2015) where they found 38.2% and 30% respectively. The higher percentage in the present study might be due to smaller and only microscopy positive sampling.

## 5.2 Sex-specific prevalence of the disease

Sexual category of animals also has influences in the occurrence of vector borne parasitic diseases. In present study revealed that male dogs carrying more prevalence of babesiosis than females, which suggested that bite wounds or blood transmission during fighting contact as possible routes of transmission for *B. gibsoni*. Male dogs most commonly associated with dog fighting or perhaps it may be due to the aggressive behavior of males, against other males especially during mating period to be dominant and therefore they get wounded and infected, or another possibility was

the hormonal status of males that may lead to high infection (Bashir, 2008; Salem and Farag, 2014). On the other hand, some reports hinted that female dogs are more prone to babesiosis than males (Singh et al., 2012; Singh et al., 2014). However, there is no difference in sex susceptibility between males and females (Martinod et al., 1986).

### 5.3 Age-specific prevalence of the disease

Age also influences the occurrence of haemoprotozoan diseases. In current study, higher susceptibility of adult dog to babesiosis was found consistent with some findings (Hornok et al., 2006; Salem and Farag, 2014). They reported, *Babesia* infection has been known to rise with the age, reaching its peak between the age of 3 and 5 years. Again *B. canis* was recorded only from the dogs above 1 year of age and the results are similar to earlier reports (Singh et al., 2012). Another study indicated that dogs from day one to two years of age were significantly more likely to test positive for the presence of *Babesia* spp than the dogs of the other ages and *B. gibsoni* is transmitted transplacentally and not by the transmammary route (Fukumoto et al., 2005; Bashir, 2008). Whereas, it has also been reported that age do not have any influence on the animals' susceptibility to the disease (Martinod et al., 1986).

### 5.4 Identification and characterization of *Babesia sp*

For several reasons, a definitive diagnosis of canine babesiosis can be difficult to achieve in the clinical background. Light microscopic examination cannot consistently differentiate species or subspecies. The lack of standardized serologic assays, the presence of cross-reactive antibodies and recent changes in the geographic ranges of several canine piroplasms have also further complicated the diagnosis of babesiosis in dogs. In the present study we detected *Babesia* organism via PCR, when compared to microscopic examination results; PCR proved the superiority and the sensitivity in the diagnosis and differentiation of *Babesia* infection in suspected dogs; however, the cost, equipment, and time may be a major limitation for the use of PCR in clinic-based practice (Irwin, 2007; Solano-Gallego et al., 2008). Again, in the present study, we describe a seminested PCR, which is specific for the diagnosis and differentiation of *Babesia gibsoni* (Asia genotype), *B. canis* subsp. *vogeli*, *B. canis* subsp. *canis* or *B. canis* subsp. *rossi.* In the seminested PCR we found *Babesia gibsoni* (Asian type) which was consistent with the earlier report published in Bangladesh (Talukder et al., 2013; Terao et al., 2015). Again in another round of

seminested PCR we found, *B. canis* subsp. *canis* for the first time in Chittagong, Bangladesh.

# Chapter VI: Conclusion

The study was performed aiming to determine the prevalence of babesiosis in stray dog and standardization of PCR-based molecular diagnosis and identification of babesiosis. Canine babesiosis was strongly associated with the age and sex of the animals where adult male dogs were more susceptible than others. The present study also revealed that PCR is more sensitive and specific than microscopic examination. *Babesia gibsoni* and *Babesia canis* subsp. *canis* was the dominant organism for canine babesiosis in this part of Bangladesh which was established by a seminested PCR and phylogeny through gene sequencing. Finally, we recommended further studies focusing identification of tick vectors along with molecular detection of organisms from tick for taking further control strategies in the study areas.

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# Appendix-1

## Data Sheet

Id.no…… Date……

## Stray dog

1. Location……..
2. Breed……
3. Age…….…….Adult/young
4. Sex……..Male/female
5. External parasite……..
7. Clinical abnormalities……..

# Appendix-2

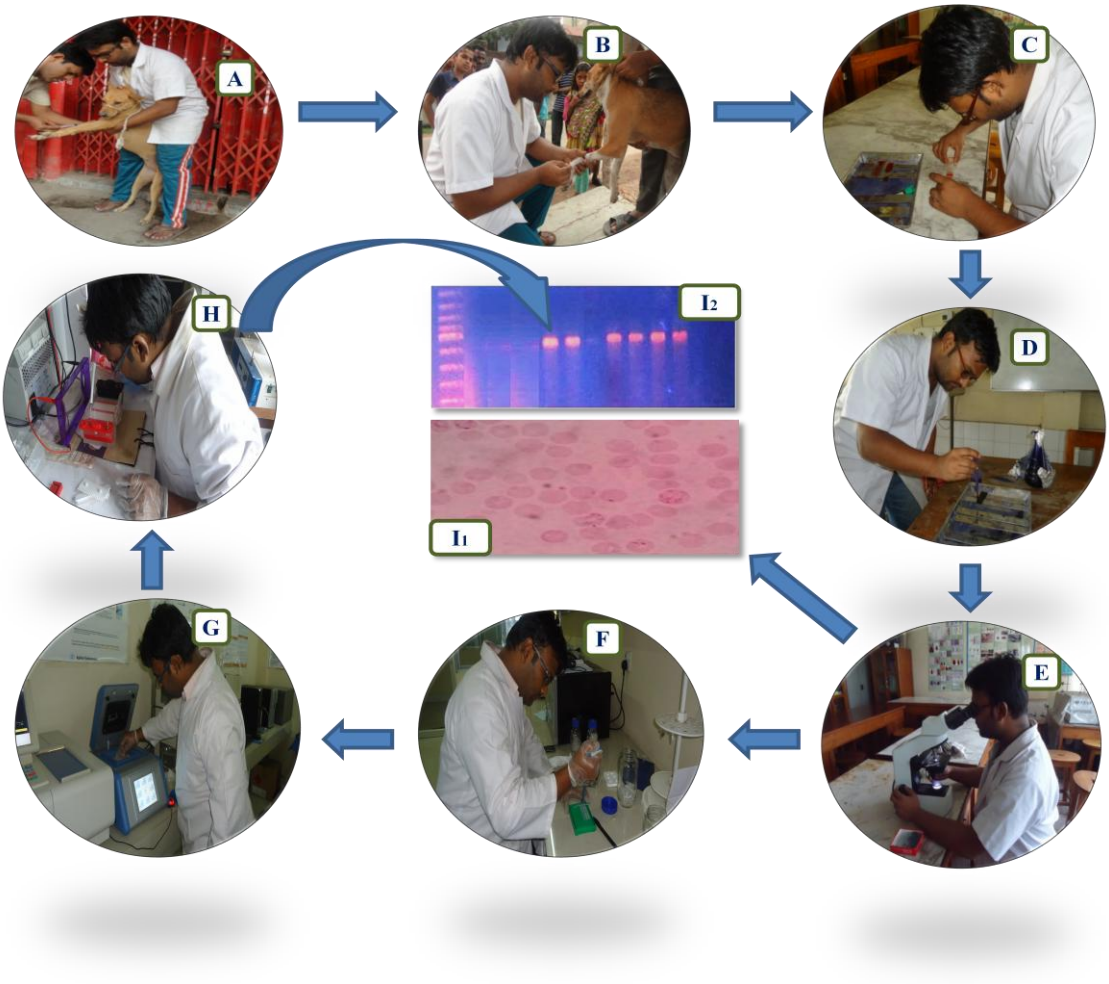


**Figure 19:** Major activities during study period.

**A.** Restraining, **B.** Blood collection, **C.** Smear preparation, **D.** Staining, **E.** Microscopy, **F.** Sample loading in PCR tube, **G.** PCR tube loading in PCR machine, **H.** Gel running **$I_1$.** Microscopic positive sample, **$I_2$.** Positive band in gel

# Brief Biodata of the Student

Md. Nur-E-Azam is a candidate for the degree of MS in Parasitology under the Department of Pathology and Parasitology, Faculty of Veterinary Medicine, CVASU. He passed the Secondary School Certificate Examination (SSC) in 2004 from Birganj Govt. High School, Dinajpur and then Higher Secondary Certificate Examination (HSC) in 2006 from Cantonment Public School and College, Rangpur. He obtained his Doctor of Veterinary Medicine (DVM) Degree in 2011 from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. He has great interest on molecular and clinical parasitology.